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Acquired Bracket Pellicle Modulation Via Exposure To Histatin 3

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Graduate Program in Orthodontics
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Abstract

Objectives: To investigate the effect of histatin 3 on the protein pellicle formation on the orthodontic bracket surface.

Methods: Our study consisted of 4 sample groups. A sample of human parotid saliva without histatin functioned as a control group. Other groups were immersed with or without histatin 3 and human parotid saliva. Each group was incubated for 2 hours in their respective substrate at 37°C. The acquired pellicle from each group was harvested, then analyzed with SDS-PAGE and LC-ESI-MS/MS.

Results: Thirty-nine proteins were identified in the control group, 18 were identified in group 2, and 21 were identified in group 3. Thirteen proteins were common to all groups. Groups immersed in histatin 3 and human parotid saliva showed an increase in the percentage of proteins exhibiting antimicrobial activities and immune response.

Conclusions: There were functional differences in the protein pellicle formed on the orthodontic bracket, suggesting that exposure to histatin 3 may alter pellicle formation. However, structural differences were limited due to redundant characteristics of salivary proteins.

Key words: orthodontic brackets, Acquired enamel pellicle, Acquired bracket pellicle, Mass Spectrometry, Protein Identification, Dental Caries, White spot lesions

Co-Authorship:

The completion of this thesis was possible due to the contribution of several individuals. It would not have been possible without their valued time and efforts, which is greatly appreciated.

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List of Abbreviations

ABP	Acquired Bracket Pellicle
AEP	Acquired enamel pellicle
aPRPs	Acidic Proline-rich proteins
BCA	Bicinchonic acid
bPRPs	Basic Proline-rich proteins
DTT	Dithiothreitol
gPRPs	glycosylated Proline-rich proteins
HPS-nH	Human Parotid Saliva
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LC-ESI-MS/MS	Liquid chromatography-electrospray ionization-tandem mass spectrometry
MS	Mass Spectrometry
MUC5B	High molecular weight mucin glycoprotein
MUC7	Low molecular weight mucin glycoprotein
PRPs	Proline-rich proteins
SDS-PAGE	Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
TFA	Trifluoroacetic Acid
XPS	X-ray photoelectron spectroscopy
WSL	White Spot Lesion

CHAPTER 1

1.1 Introduction

Active carious lesions is an area of concern among practitioners as it is a common adverse effect of orthodontic treatment. Demineralization in early enamel caries gives the lesion a chalky white appearance. This visible defect in the enamel is known as a white spot lesion (WSL). The reported prevalence of WSL in the literature is widely variable,[1] however most studies report the incidence of new clinically visible WSL occurring during orthodontic treatment to range between 30-70% of patients.[1, 2] The caries process requires certain bacteria within the biofilm which produce acids as a byproduct of its metabolism. These acids contribute to the demineralization of enamel which is the contributing factor in the chalky appearance of WSL.[3]

The acquired enamel pellicle (AEP) is a thin film that forms on the enamel surface. The AEP primarily consisting of salivary proteins provides an interface between the enamel and the oral environment and as such plays a critical role in subsequent biofilm formation, demineralization, remineralization and lubrication.[4] It is recognized that specific proteins/peptides have a greater affinity for enamel than others.[5]

Several studies have shown that bacterial growth on the surface of orthodontic brackets varies in quality and quantity depending on the material composition of the bracket.[6–8] Although many studies have focussed on bacterial adhesion to orthodontic brackets,[6, 9] few have focused on the pellicle formation on the orthodontic bracket. The quality and quantity of proteins in pellicle formation on orthodontic materials differs depending on material composition.[10, 11]

Using liquid chromatography electrospray ion tandem mass spectrometry (LC-ESI-MS/MS) over 130 different proteins in the human AEP have been identified.[12, 13] One notable protein found in the AEP is histatin. Histatin is a low-molecular-weight salivary protein excreted by the major and minor salivary glands,[14, 15] with many properties including antifungal and antimicrobial activity.[14, 16, 17] Histatin has also been previously been identified as part of the pellicle formed on the metallic orthodontic bracket.[11]

In addition to antimicrobial and antifungal properties, histatin has also shown a functional effect in protection against demineralization. In an *in vitro* study, enamel samples were subjected to a pH of 4.5. Samples that were subjected to histatins showed both a reduction in phosphate and calcium loss compared to the control which received no protein treatment[14]. A reduction in the loss of both calcium and phosphates in enamel subjected to an acidic environment shows that histatins also have the potential to provide some level of protection against acidic attack and may play a functional role in both buffering capacity and mineral homeostasis of the acquired enamel pellicle.

Microbial metabolism and demineralization are the causative factors resulting in enamel caries. Therefore, the buffering capacity, mineralization and antimicrobial activities of histatin target the protein worthy of investigation in its role in the acquired pellicle which forms on the orthodontic bracket.

Our objective is to investigate how exposure to the histatin 3 peptide will affect the protein pellicle formed on the metallic orthodontic bracket.

1.2 Literature Review

1.2.1 Orthodontic treatment and Dental Caries

Caries development is a process that requires cariogenic bacteria, time and fermentable carbohydrates. Streptococcus mutans and lactobacillus are primary contributors to the caries process through the acidic by-product in the metabolism of fermentable carbohydrates resulting in demineralization of the enamel. [18] Early enamel caries is first clinically evident as a white spot lesion. The white chalky appearance of the enamel is due to the differential refraction of light resulting from the demineralized surface and subsurface of the enamel.[2]

The etiology of white-spot lesions in orthodontic patients is multifaceted. Orthodontic brackets, wires and auxiliaries provide a greater surface area for the adhesion of plaque. Their irregular shapes also make mechanical plaque removal more difficult as well as decreasing the ability of self-cleansing properties provided by saliva, lips, tongue and cheeks.[2] This increase in plaque around orthodontic appliances results in a lower pH which drops below the threshold for remineralization and decalcification occurs.[19] The decrease in pH is caused by the increased colonization of acidogenic bacteria such as Streptococcus mutans during orthodontic treatment.[3, 20]

Several studies have been done regarding microbial adhesion to various types of orthodontic brackets,[6, 9, 10, 21] and it is evident that there are significant differences in the microbial adhesion on various orthodontic materials. The initial attachment plays an important factor governing further colonization.[21] Further colonization of microorganisms is dependent on the pellicle that is

first formed on the surface of the orthodontic appliance.[8] Certain salivary proteins such as cystatins, acidic PRPs, amylase, sIgA, and MG2 are known to act as receptors for bacterial adhesion.[8] However, little is known regarding the modulation of the oral environment and its impact on the subsequent adhesion of various proteins to the orthodontic bracket.

1.2.2 Saliva characteristics

1.2.2.1 Saliva definition and composition

Salvia is a clear, slightly acidic (normal pH is 6.8) mucoserous exocrine secretion. It is composed of 99% water and a variety of electrolytes including sodium, potassium, calcium, magnesium, and phosphates. Immunoglobulins, proteins, enzymes, mucins and nitrogenous products such as urea and ammonia are also present.[22] However, composition varies according to many factors, including gland type.[23]

1.2.2.2 *Whole saliva*

Whole saliva or oral fluid is a complex mix of fluids (both stimulated and unstimulated) from major salivary glands (parotid, sublingual, and submandibular) and minor salivary glands, gingival crevicular fluid, non-adherent oral bacteria and food debris, and traces of introduced chemicals or medicaments.[22]

1.2.2.3 *Salivary glands*

The major salivary glands include the paired parotid, submandibular and sublingual glands. Numerous minor salivary glands are located in the oral cavity with the exception of the anterior hard palate, the dorsum of the tongue and the gingiva.[24] When compared to the thousands of proteins identified in the major salivary glands only 56 proteins were identified in the minor salivary gland proteome.[25] Although it has been shown that minor salivary glands contribute only about 10% of the total volume of human saliva,[26] minor salivary glands are significant in their role in oral health, largely due to their proximity to mucosal surfaces and the enriched number of mucins and immunoglobulins.[25]

1.2.2.4 *Salivary flow*

Unstimulated individual salivary flow rate is highly variable, however, the average has been reported to be 0.3 mL/min, while the accepted minimal minimal flow rate is 0.1mL/min.[22] There are both daily and annual ebbs and peaks in flow rate. Salivary flow rate while sleeping is nearly zero and peak occurs at times of stimulation periods. Additionally, circadian variation also occurs with protein electrolyte concentrations.[27] Annually peak salivary flow rates are highest in the winter, and lowest in the summer months.[22] Percentage contributions of the different salivary glands are also dependant on stimulation. Percent contribution of unstimulated flow are as follows: 20% from parotid, 65% from submandibular, 7% to 8% from sublingual, and less than 10% from numerous minor salivary glands. Under stimulation the parotid gland increases in percent contribution to more than 50%.[22]

There are three specific triggers for salivary flow: mastication, taste, and smell. These triggers are what signal the nuclei in the salivary centre within the medulla which controls secretion.[22] Secretion is a two-stage process,[28] where it is initially formed in the acing lumen, then the salivary ducts modify the primary fluid by removing sodium and chloride, and adding potassium and bicarbonate to produce a hypotonic fluid that enters the oral cavity.[29] The duct system cells found in salivary glands are classified as intercalated, striated, and excretory. Intercalated duct cells connect the acinar secretions to the rest of gland. Striated duct cells are second in the network followed by the excretory duct cells. The striated duct cells begin to remove the sodium from the original isotonic fluid, which is continued by the excretory duct cells. Additionally the excretory duct cells secrete potassium. The intercalated cells are not involved in electrolyte regulation. Finally, accumulating fluid is excreted by contracting myoepithelial cells which wrap around the acinar cells. [22]

1.2.2.5 *Type of secretion*

Acinar cells can be divided into two general types: serous and mucous. It is therefore the type(s) of acinar cells contained within the gland that determine the secretion classification. Secretion can be classified as serous, mucous or mixed. The secretions of the parotid glands are classified as serous, whereas the secretions of the submandibular and sublingual glands are classified as mixed. Serous acinar cells significantly outnumber the mucous acinar cells in the submandibular gland, while the mucous acinar cells outnumber the serous acinar cells in the sublingual gland. Minor salivary gland secretion is mostly mucous.[30]

Parotid saliva contains amylase, proline-rich proteins and agglutinins with small amounts of cystatins, lysozymes, and extra parotid glycoproteins.[22] Furthermore, the parotid glands are the main source of amylase in whole saliva, where no mucins found in whole saliva come from the parotid gland.[24] Sublingual saliva contributes high amounts of both types of mucins (MG1 and MG2) and lysozymes, whereas, submandibular saliva contains the largest concentrations of cystatins. Palatine secretions have higher concentrations of MG1 mucins and relatively high amounts of amylase.[31]

There are a large number of factors that contribute to the total salivary protein concentration as well as the relative proportions.[24] These include the age of the subject,[32] the circadian rhythm, the flow rate, and the length of time since the glands were last stimulated, and the nature of stimulation.[24]

1.2.2.6 *Role of saliva*

Saliva functions to maintain an appropriate ecological balance in the oral cavity and maintain oral health. These functions have been separated into five major categories: (1) Lubrication and protection, (2) Buffering action and clearance, (3) maintenance of tooth integrity, (4) antibacterial activity, and (5) taste and digestion.[22, 33, 34]

The seromucous coating provided by saliva, lubricates and protects oral tissues from irritants such as proteolytic and hydrolytic enzymes produced in the plaque, exogenous chemicals and desiccation. Lubrication is largely provided by mucins secreted by the minor salivary glands.[22]

Lubricating effects of mucins also contribute to the functions of mastication, speech and swallowing.[35] Mucins also serve to modulate the adhesion of microorganisms to oral tissue surfaces, thus providing an antibacterial effect. [22]

Bicarbonate, phosphate, urea, and amphoteric proteins and enzymes are components of saliva that provide the buffering action and clearance. The main buffering action comes from bicarbonate which diffuses into plaque and generates ammonia to form amines serving as a buffer to neutralize acids.[36] Histidines provide 90% of the non-bicarbonate buffering action. Additionally, urea releases ammonia after being metabolized by plaque which consequently increases the pH.[22]

Demineralization and remineralization is a cyclical process in which saliva plays a critical role in homeostasis. Demineralization occurs when acids diffuse through the plaque and enamel pellicle resulting in crystalline dissolution, a process in which dissolved minerals diffuse out of the tooth structure. Crystalline dissolution occurs at a pH of 5 to 5.5. It is the buffering capacity of saliva that increases the subsequent pH inhibiting demineralization.[22] Remineralization occurs when minerals lost during crystalline dissolution are replenished. Supersaturation of minerals such as calcium, phosphate and fluoride in saliva are critical to this process.[22] Salivary proteins contained in the acquired enamel pellicle (AEP) such as histatins, statherin and mucins play contributing roles in protecting enamel from demineralization.[4]

Antibacterial activity includes both immunologic and non-immunologic agents. Immunologic agents secreted in saliva include IgA, IgG, and IgM, with IgA being the largest component. Non-immunologic components include, selected proteins, mucins, peptides, and enzymes[22]

1.2.2.7 *Proteins*

Many proteins found in saliva are multifunctional. Major salivary functional properties include: antibacterial, antiviral, antifungal, buffering, digestion, mineralization, and lubrication (Table 1).[37] Protein functions can be redundant and also have both protective and detrimental properties.[37]

Table 1 - Multifunctional characteristics of the major salivary families

Function	***Salivary Protein***
Antibacterial	Amylase, cystatins, histatins, mucins, peroxidases, statherins
Antiviral	Cystatins, mucins
Antifungal	Histatins
Buffereing	Carbonic anhydrases, histatins
Digestion	Amylases, mucins
Mineralization	Cystatins, histatins, proline-rich proteins, statherins
Lubrication	Amylases, cystatins, mucins, proline-rich proteins, statherins

Studies also have demonstrated that functional relationships stemming from complexes between different molecules can provide additional functions over and above the individual molecules comprising the complex.[37] Protein families that demonstrate antibacterial, buffering, and mineralization functions are of particular interest in their role in the prevention of dental caries and white spot lesions.

Proline-rich proteins (PRPs):

Proline-rich proteins can be divided into three groups: (1) acidic (aPRPs), (2) basic (bPRPs), and (3) glycosylated basic (gPRPs). They account for more than 60% in weight of the total salivary proteome,[38] and are major components of parotid and submandibular saliva.[39] The primary structure of the acidic proline-rich proteins is unique and they do not belong to any known family of proteins. An important role of acidic PRPs is the ability to inhibit the formation of hydroxyapatite through modulation of oral calcium ions[38]. Glycosylated PRPs express some lubricating properties while basic proline-rich proteins protect against the toxic effects of tannins.[38]

Histatins:

Histatins are small peptides which have a high number of histidine residues in their structure.[38] Histatins have demonstrated multifunctional properties; one of which is the ability to kill pathogenic yeasts.[14, 15] It has also been shown that histatins have antibacterial properties against a variety of bacteria.[14] Results from Siqueira *et al* showed that all 3 naturally occurring histatins (ie. histatin 1, histatin 3 and histatin 5) are found in the acquired enamel pellicle and have the potential for protection against demineralization.[14] Due to their multifunctional role, histatins play an important role in the maintenance of a balanced oral microbial ecology.[40] Histatins are found in greater concentrations in pure glandular secretions compared to that of whole saliva. It is suggested that the difference in concentration is likely due to high proteolytic activity causing rapid degradation of histatin in whole saliva.[40] Histatins have also been described as metal-bind-

ing peptides[41] and have been cited as part of the pellicle formed on metallic orthodontic brackets.[11]

Statherin:

Statherin is another protein that is found in the pellicle formed on both enamel, and the metallic orthodontic bracket.[11]. Statherin is involved in calcium ion homeostasis within the oral cavity through inhibiting primary and secondary calcium phosphate precipitation, thereby supersaturating the saliva and contributing to remineralization.[38, 42] Statherins have also been found to exhibit antimicrobial activity.[43]

Cystatins:

Salivary cystatins include cystatin S, SN and SA, all of which belong to family 2 of cystatins.[38, 44] As inhibitors of cysteine proteinases, cystatins play a protective role from pathogens and lysosomal cathepsins.[38] While cystatins also inhibits calcium phosphate precipitation, it does so at a tenth that of statherin.[45] Cystatins are included as one of the most abundant proteins in the acquired enamel pellicle.[4]

Amylases:

The most abundant salivary protein found in whole saliva is salivary amylase, which accounts for 20% in weight of salivary proteins.[38, 40] Salivary α-amylase is mainly synthesized in the parotid gland, but α-amylase is also synthesized in the pancreas aiding in digestion of dietary

starch.[40] Salivary amylase contributes to food digestion through hydrolysis of starch to glucose and maltose.[46, 47] Salivary amylase has shown to have an inhibitory effect in the growth of *Legionella pneumophila*, and *Neisseria gonorrhoea.*[48] Additionally, salivary amylase have receptors that bind to strains of streptococcus, and can facilitate clearance of bacteria from the oral cavity.[48]

Mucins:

Mucins are gel-forming mucous glycoproteins with a high molecular weight that cover the epithelial surfaces of the gastrointestinal, respiratory, and reproductive tracts.[40] Intra-orally mucins are secreted by the submandibular and sublingual glands, as well as the minor salivary glands and are the third most abundant group of proteins in whole saliva.[40, 49] Salivary mucins have either a high molecular weight (MG1) or a low molecular weight (MG2). Major components of the salivary mucin family include MUC5B (part of the MG1 family) and MUC7 (part of the MG2 family).[25, 40] Mucins function to protect the epithelium from desiccation, mechanical injury, and microbial attack. Additionally, mucins contribute to the salivary characteristics that aid in mastication, swallowing, speech, and protecting the teeth from abrasive forces.[40] Mucins also serve to modulate the microflora by favouring the attachment and/or clearance of certain microorganisms.[35] They have been shown to bind to several strains of *S. Mutans* and *A.* a*ctinomycetemcomitans.*[50] Furthermore, mucins form complexes with other salivary proteins including histatins, amylase, statherin, and PRPs enhancing or diminishing the activity of either one.[50]

1.2.3 Acquired Enamel Pellicle

1.2.3.1 *Definitions*

The acquired enamel pellicle (AEP) is a thin acellular film that forms on tooth surfaces upon exposure to the oral environment.[4] In its initial stages (within seconds of exposure to whole saliva), the pellicle increases in thickness to 10 to 20 nm within a few minutes and remains stable for approximately 30 minutes.[4] Salivary proteins that have a high affinity for hydroxyapatite initiate the process through electrostatic interactions with the enamel surface.[4] Protein-protein interactions contribute to further protein adsorption and an increase in AEP thickness. After 30 to 90 minutes the pellicle thickness reaches a plateau of 100 to 1,000 nm depending on the location within the oral cavity.[4] The AEP plays a functional role in the maintenance of oral health by regulating lubrication, demineralization, and remineralization. The AEP also contributes to antimicrobial activity by influencing the subsequent composition of the microflora.[4] Additionally, individual proteins such as histatins have shown to exhibit antimicrobial properties as described earlier.

1.2.3.2 *Proteomic Analysis*

Previously, the molecular structure of the AEP has been difficult to assess due to the limited quantities of *in vivo* formed pellicle which can be harvested. The amount of protein collected per tooth surface has been shown to amount to approximately 0.5 - 1 µg.[51] Developments in both the harvesting methodology and new mass spectrometry techniques have increase the ability for proteomic analysis of the AEP.[12] Proteomics using 2-D gel electrophoresis have been utilized, how-

ever they are limited by their dependence on protein separation, the visualization of proteins in the gel, and the labor-intensive processing of excised protein spots.[13]

Mass spectrometry has the ability to generate a protein profile and sequence information, thus allowing a direct analysis of complex biological samples. More recently, “shotgun” proteomic methods using mass spectrometry have gained interest due to the ability to reduce sample handling time and also eliminate the need for processing individual proteins.[13]

1.2.3.3 *Composition and Function of AEP*

The functions of the AEP include neutralization of acid produced by oral bacterial and providing lubrication, thus protecting the enamel from abrasive forces.[13, 52] The AEP also dictates the composition of the initial microbial tooth colonizers which then provide attachment substrates for subsequent colonizers, ultimately influencing the succeeding stages of biofilm formation.[53]

Using mass spectrometry, 130 pellicle proteins were previously identified in *in vivo* AEP studies. According to their possible role in AEP structure formation, three main groups forming the AEP were identified in past research.[13] The calcium binding group made up 17.5% of the identified AEP proteins. Among these were the S100 calcium binding protein family and members of the annexing family. They are considered to be pellicle precursor proteins due to their interaction with calcium ions on the enamel surface.[13] The second group consisted of proteins that show a high tendency to bind phosphate ions. These proteins include: elongation factor 2 and myosin-9. These proteins along with calcium binding proteins are considered to form the primary portion

layer which adsorbs to the enamel surface, as enamel is composed of calcium phosphate salts (hydroxyapetate).[13] The third group identified in the literature[13] are proteins that have an interaction with other protein, such as MUC 5B, which are suspected to be involved in the formation of the successive protein layers.[13]

AEP proteins were also grouped according to their biological functions such as inflammatory response, antimicrobial activity, immune defence, buffer capacity, lubrication, and remineralization capacities.[13, 54] Proteins that are members of the calgranulin and annexing families were identified in the group involved in remineralization. Proteins identified that play a role in host immune response or exhibited antimicrobial activity included cystatins(S, SA, A, B, D), lysozyme, lactotranserrin, myeloperoxidase, calgranulin A, and calgranulin B. Albumin, aplpha-1-antitrypsin, cathepsin G, complement C3, and myeloperoxidase were also identified in the AEP, which also have been identified as strong biomarkers for periodontal disease. These inflammatory biomarkers constituted 13% of the total number of AEP proteins.[13]

Previous research also analyzed the proteins with respect to their origins.[13] Only 14.4 % of all proteins found in the AEP were derived from exocrine salivary secretions. The majority of the proteins identified in the AEP were of a non-exocrine origin including cells (67.8%) and serum (17.8%). It is thought that as more salivary gland proteins are identified in future research, some of the AEP proteins identified as being derived from cells or serum are actually true salivary gland derived proteins.[13]

1.2.3.4 *Salivary Pellicles on Orthodontic Brackets*

Salivary pellicles are not limited to enamel, but adhere to surfaces exposed to saliva in the oral environment such as the oral mucosa, [55] orthodontic brackets and other dental appliances,[10] restorations and titanium implants.[56] As with the AEP, the salivary pellicle on other surfaces will act as determinant for microbial colonization. Bacterial adhesion to orthodontic brackets is a result of lectin-like reactions,[57] electrostatic interactions, and van der Waals forces.[58] These reactions are dependent on the composition of the surface material, thus affecting the composition of proteins found in the pellicle and the subsequent micro-flora.[8, 21] When comparing orthodontic bracket material, it has been found that the affinity for Streptococcus mutans was significantly lower on metallic brackets compared with other materials.[59] Ahn *et al* found that low molecular weight mucin, alpha-amylase, secretory IgA, acidic proline-rich proteins, and cystatins adhered to all types of brackets, but that the composition amino-acids in the pellicles differed between bracket type as well as the subsequent micoflora.[8] Lee reported that the pellicle formed on elastic ligatures was similar to that of metallic orthodontic brackets, but differed when compared to bonding resin. Additionally, Lee found that the degree of adhesion of cariogenic streptococci was significantly higher for bonding adhesives than for bracket materials.[60]

1.2.5 Two-Dimensional Gel Electrophoresis

Chemical and mechanical means have been used previously to identify proteins found in the AEP. However, using this method, only 18 proteins were identified.[13, 61] First introduced in 1975 by O'Farrell and Klose, two-dimensional electrophoresis allows proteins to be identified using a

two-dimensional analysis. In the first dimension, using electrophoresis, proteins are separated linearly according to their isoelectric point. Molecules are then separated at 90 degrees (in the second dimension) from the first electropherogram according to their molecular mass. However, before separating the proteins by mass, they are treated with sodium dodecyl sulphate (SDS) along with other reagents such as 30% polyacrylamide, water, Tris, ammonium persulphate, and TEMED (SDS-PAGE) which denatures the proteins, unfolding them in to straight molecules.[62, 63] Using proteomics technologies such as a two-dimensional gel electrophoresis, followed by mass spectrometry the number of identifiable proteins increased to 200.[13, 64] However, it has been pointed out that the general limitations of 2-D PAGE are the dependence of protein separation, the visualization of proteins in the gel, and the labor-intensive processing of excised protein spots.[13, 65]

1.2.6 ElectroSpray Ionization Tandem Mass Spectrometry (LC-ESI-MS/MS)

Mass spectrometry (MS) has gained popularity in proteomics due to its ability to handle the complexities of the proteome and has become an indispensable tool in proteomics for the interrogation of protein expression, protein interaction, and protein modification.[66] Mass spectrometry can generate a protein profile and sequence information allowing the characterization of peptides down to the femtomole (10^{-15}) level.[13] Proteins extracted from biological samples can be analyzed by bottom-up or top-down methods. In the bottom-up approach, proteins in complex mixtures can be digested into a collection of peptides, which are then separated by multidimensional chromatography on-line coupled to tandem mass spectrometric analysis. This is also

known as the shot-gun approach.[66] Alternatively, protein mixtures can be separated before digestion, followed by direct peptide mass fingerprinting-based acquisition or further peptide separation on-line couple to tandem mass spectrometry.[66] Using the top-down approach, proteins are fractured and separated into pure single proteins or less complex mixtures, followed by off-line static infusion of sample into the mass spectrometer for intact protein mass measurement and intact protein fragmentation.[66]

1.2.7 Metallic Bracket Composition

A 2018 XPS analysis of American Orthodontics Mini Master metallic brackets demonstrated a variation in the percentage of identified elements between brackets from the same manufacturer. Identified elements included: silver, boron, chromium, copper, iron, nitrogen, sodium, niobium, oxygen, palladium, sulfur and silicon.[11]

CHAPTER 2

2.1 MATERIALS AND METHODS

2.1.1 Study design

Four groups were considered in this study design. Each group consisted of 10 metallic orthodontic twin brackets brackets from American Orthodontics. Group 1, a control with orthodontic brackets incubated in human parotid saliva without histatin (HPS-nH) only. Group 2, brackets were incubated first in HPS-nH, followed by incubation in histatin 3. Group 3, brackets were incubated first in histatin 3, followed by HPS-nH without histatins. Group 4, brackets were incubated in histatin 3 only to evaluate the binding of histatin 3 to metallic orthodontic brackets. Groups 1-3 were evaluated for potential acquired bracket pellicle modulation via exposure to histatin 3. The design was triplicated and repeated on two separate days. Proteins/peptides identified by mass spectrometry that were identified in two of the three triplicate samples were included in the data analysis.

2.1.2 Human parotid saliva without histatins

Human parotid saliva (HPS) was previously collected by Dr. Walter Siqueira and kept frozen at -20°C. Samples were collected with the use of a Carlson-Crittenden device positioned over Stenson's duct into a polypropylene graduated cylinder chilled on ice. Histatins were immediately removed using a two-step procedure, first using zinc precipitation of histatins, followed by final purification using reversed-phase high-performance liquid chromatography. Total protein con-

centration was then determined using the bicinchoninic acid (BCA) assay (Pierce Chemical, Rockford, IL, USA). The standards in various dilutions of a known concentration of bovine serum albumin (BSA) were prepared along with the working reagent as per the protocol outlined by Pierce Chemical. The mean protein concentration of the HPS-nH was 1.33 mg/mL.

2.1.3 Bracket preparation

Forty orthodontic metallic twin central maxillary incisor brackets from American Orthodontics were divided into four groups. Ten brackets in each group were placed using sterile thumb forceps in a 1.8 mL polypropylene microcentrifuge tube with 1.0 mL ultra-pure MilliQ water and were cleansed using sonication for 15 minutes. Ultra-pure Milli-Q water was used throughout all experiments to avoid water contaminants.

2.1.4 Incubation in HPS-nH and histatin 3

Immediately after the brackets in each group were cleaned, they were transferred using forceps from the polypropylene microcentrifuge tube to their respective wells in a 24 well culture plate. Wells from groups 1 and 2 each contained 800 mL of ultra-pure MilliQ water with 200 µg of proteins from HPS-nH. Wells from groups 3 and 4 each contained 800 mL of ultra-pure MilliQ water with 50 µg of Histatin 3. The brackets were then gently agitated as they were incubated in their respective wells for 2 hours at 37°C. Immediately after incubation, the brackets from each group were removed from their respective wells using sterile forceps and washed in MiliQ water to remove any weak binding proteins. Brackets from groups 1 and 4 were then placed in 1.8 mL

low binding tubes for harvesting. Brackets from groups 2 were placed in new wells containing 800 mL of ultra-pure MilliQ water with 50 μg of Histatin 3. Brackets from groups 3 were placed in new wells containing 800 mL of ultra-pure MilliQ water with 200 μg of proteins from HPS-nH. Groups 2 and 3 were then gently agitated while incubating in their respective wells for 2 hours at 37°C. Immediately following incubation brackets from each group were removed from their respective wells using sterile cotton forceps and washed in MilliQ water to remove any weak binding proteins.

2.1.5 Harvesting of in vitro Acquired Bracket Pellicle

The acquired bracket pellicle (APB) was harvested by placing each group of brackets in a 1.8 mL low binding tube with 300 μL of a harvesting solution containing 80% Acentonitrile, 19% MilliQ, and 0.1% Trifluoroacetic acid (TFA) and sonicated for 5 min. After sonication the buffer was extracted and placed in a 1.5 mL low binding tube. This process was repeated three times. The 1.5 mL polypropylene microcentrifuge tubes containing the harvested acquired bracket pellicle in the 900 μL of harvesting solution were then concentrated by a rotary evaporator. The total protein concentration of the harvest ABP for each group was determined using the micro bicinchoninic acid assay (μ-BCA)(Pierce Chemical, Rockford, IL, USA). The standards in various dilutions of a known concentration of bovine serum albumin were prepared along with the working reagent as per the protocol outlined by Pierce Chemical.

2.1.6 Preparation of aliquots

Due to the relatively low amount of protein contained in the acquired bracket pellicle, the experiment was repeated on a different day to obtain the protein amounts necessary for the gel SDS PAGE and mass spectrometry. The dried samples from both days containing known amounts of protein were re-suspended in ultra-pure MilliQ water and pooled within their respective groups. Aliquots were prepared containing 15 μg of protein from each group for the 2-D gel electrophoresis. The remaining protein from each group was prepared for mass spectrometry.

2.1.8 SDS PAGE

SDS PAGE gel with 12% acrylamide was carried out in all experiments. Samples were mixed with the sample buffer (Appendix 1) and heated in boiling water for 5 min. After cooling to room temperature each sample was loaded in a separate well. From left to right, the first well was loading with 5 μL of protein standard (Precision Plus Protein™ All Blue Prestained Protein Standards, Bio-Rad, California, USA), the second was loaded with a sample from our original parotid saliva without histatin, the third well was loaded with a sample of the harvested pellicle from group 1 (HPS-nH), the forth well was loaded with a sample of the harvested pellicle from group 2 (HPS-nH followed by histatin 3), the fifth well was loaded with a sample of the harvested pellicle from group 3 (histatin 3 followed by HPS-nH), and the sixth well was loaded with a sample of the harvested pellicle from group 4 (histatin 3 only). Protein separation was reached at 110V for 1.5 hrs. Following the electrophoresis, the gels were removed and placed in the

Coomassie blue staining solution (Appendix 1) for 1 hour and then destained in destaining solution (Appendix 1) until the bands were clearly visible.

2.1.7 In-solution Digestion and Preparation for Mass Spectrometer

Dried samples with varying amounts of pellicle protein remaining were re-suspended in 50 µL of a solution containing 4M Urea, 10mM DTT and 50mM ammonium bicarbonate at a pH of 7.8 and incubated for 1 hour at room temperature. Afterwards, 150 µL if 50 mM ammonium bicarbonate was added to each sample, followed by 2% (w/w) trypsin (Promega, Madison, WI, USA). Samples were then incubated at 37 °C for 16 hours and subsequently dried in a rotary evaporator.

Samples were desalted by means of a Zip Tip_{C18} (Millipore, Bedford, MA, USA) and the eluted portion was dried, resuspended in 15 µL of 97.5 % H_2O/2.4% acetonitrile/0.1% formic acid and then subjected to reversed-phase LC-ESI-MS/MS.

2.1.9 Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-MS/MS)

After trypsinization, samples were subjected to nanoscale LC-ESI-MS/MS. Mass spectrometric analysis was carried out with nano-HPLC Proxeon (Thermo, San Jose, CA, USA), which allows in-line liquid chromatography with the capillary column, 75 µm x 10 cm (Pico TipTM EMITTER, New Objective, Woburn, MA) packed in-house using Magic C18 resin of 5 µm diameter and 200 Å pores size (Michrom BioResources, Auburn, CA) linked to mass spectrometer

(LTQVelos, Thermo Scientific, San Jose, CA, USA) using an electrospray ionization in a survey scan in the range of m/z values 390–2000 tandem MS/MS. The nano-flow reversed-phase HPLC was developed with linear 80 minutes' gradient ranging from 5% to 55% of solvent B (97.5% acetonitrile, 0.1% formic acid) in 65 minutes at a flow rate of 300 nl/min with a maximum pressure of 280 bar. Electrospray voltage and the temperature of the ion transfer capillary were 1.8 kV and 250 °C respectively. Each survey scan (MS) was followed by automated sequential selection of seven peptides for collision-induced dissociation (CID), with dynamic exclusion of the previously selected ions.

2.1.10 Data Analysis

The obtained MS/MS spectra were searched against human protein databases (Swiss Prot and TrEMBL, Swiss Institute of Bioinformatics, Geneva, Switzerland, http:// expasy.org/sprot) using SEQUEST (PROTEOMS Discover 3.0, Thermo, San Jose, CA, USA). Search results were filtered for a false discovery rate of 1% employing a decoy search strategy utilizing a reverse database. Each sample is analyzed four consecutive times by the mass spectrometry, in order to have a positive identification of proteins, the same protein passing the filter score need to be identified from at least three different MS analysis from the same group in a total of four MS analyses per group.

2.1.11 Bioinformatics Characterization of Identified Proteins

The identified proteins were classified based on the their biological function, and chemical characteristics using data from UniProt (http://uniprot.org) assessed in November 2018.

2.2 RESULTS

Inclusion criteria required proteins identified by mass spectrometry to be present in two of the three triplicates for each group (Figure 1). The group which brackets were incubated in parotid saliva only (group 1) had a total of 39 proteins which were common in at least two of the three samples; the group which bracket were incubated in parotid saliva first and then in histatin 3(group 2) had a total of 18 proteins which were common in at least two of the three samples; and, the group which was incubated in histatin 3 first, then parotid saliva (group 3) had a total of 21 proteins which were common in at least two of the three samples (tables 2-4).

Figure 1 - Summary of the distribution of proteins identified from the sample preparations for group 1, group 2 and group 3

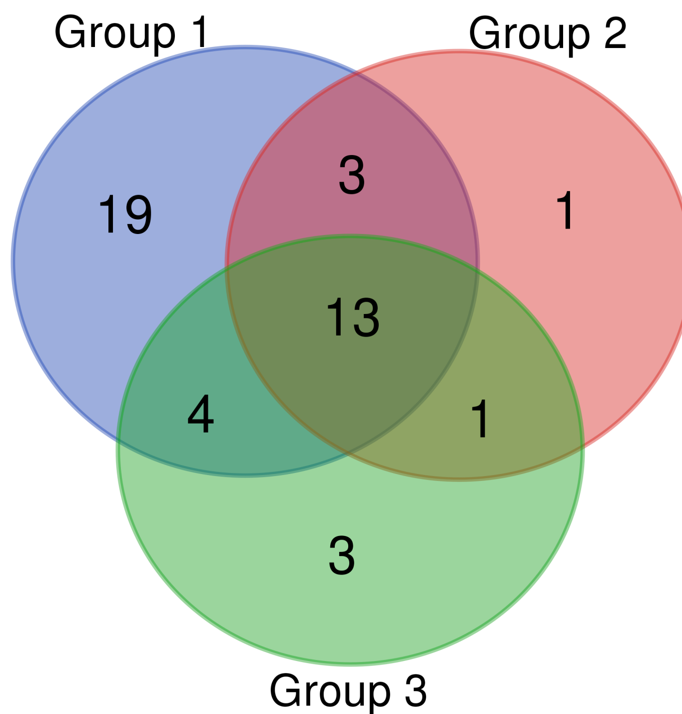

Figure 2 - Summary of the distribution of proteins identified in each of the three groups

Comparing the data from all three groups there were 44 proteins found (Figure 2). Among these proteins only 13 were common to all three groups. Four proteins were common when comparing groups 1 and 3, three proteins were common between groups 1 and 2, and only one protein was common to groups 2 and 3 (Figure 2).

Harvested pellicles obtained from each sample were retained for analysis using SDS-PAGE. SDS-PAGE was used for each of the triplicate samples and the three groups were compared (Fig 3-5).

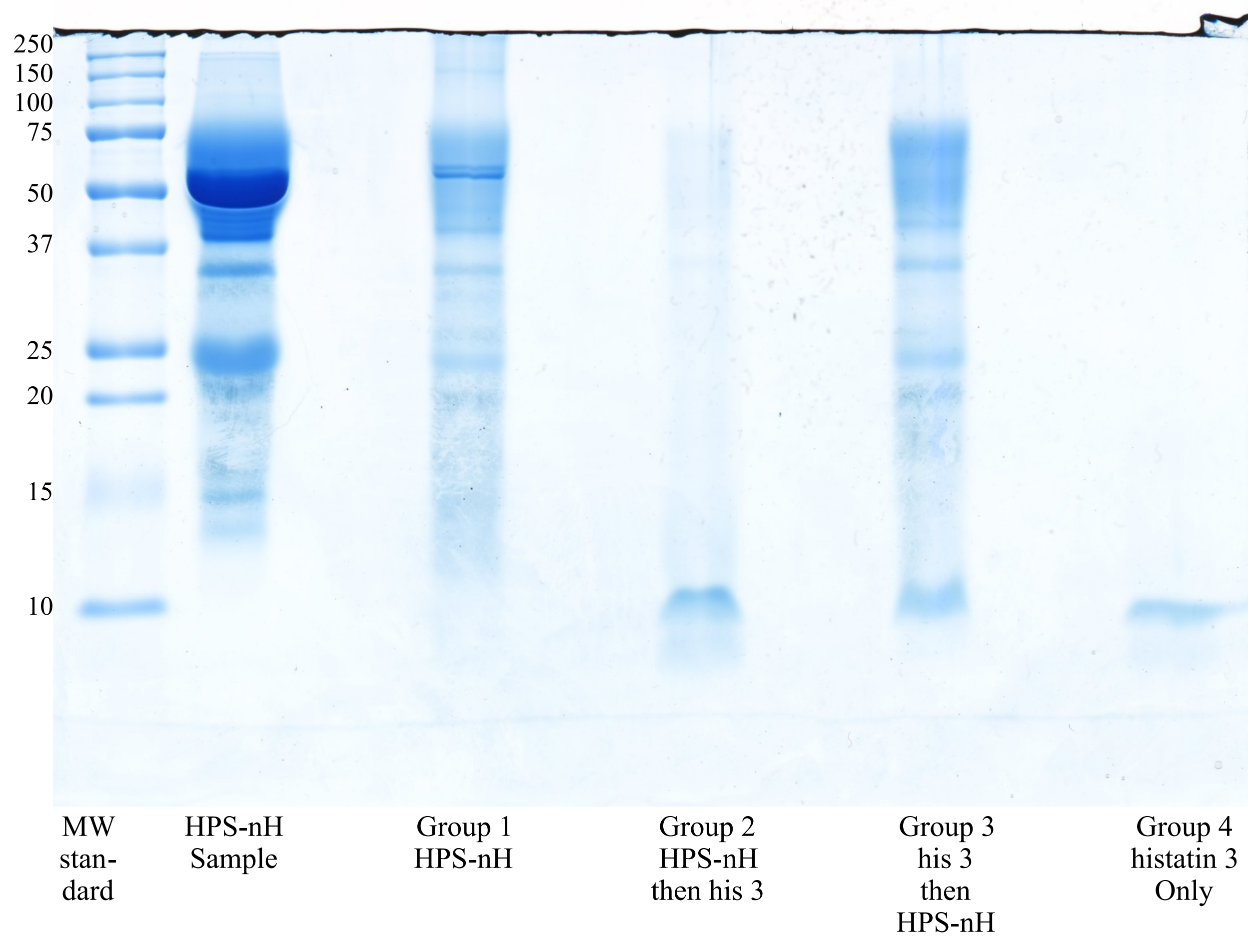

Figure 3 - Assessment of the first sample of groups 1, 2, 3 and 4 compared to original parotid saliva.

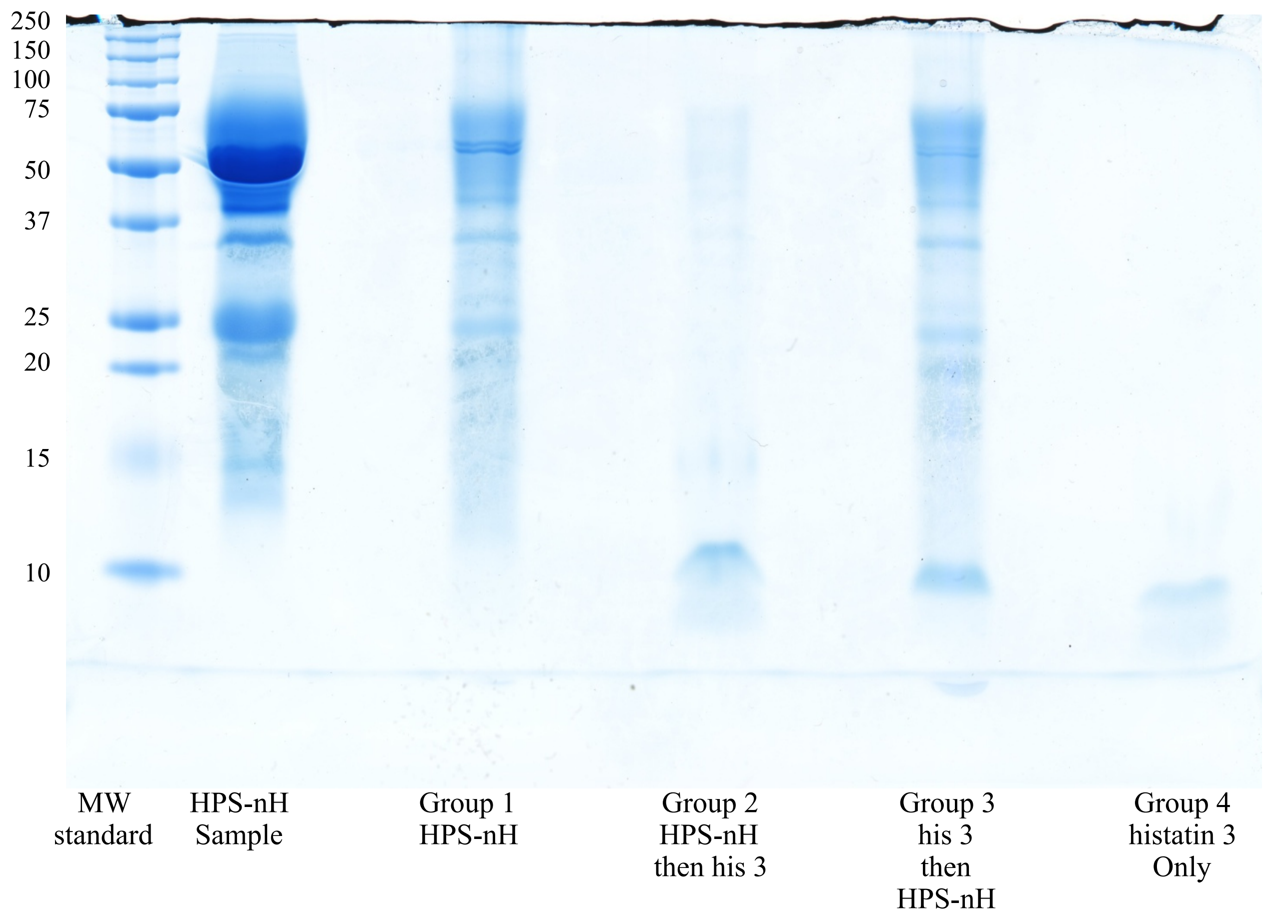

Figure 4 - Assessment of the second sample of groups 1, 2, 3 and 4 compared to original parotid saliva.

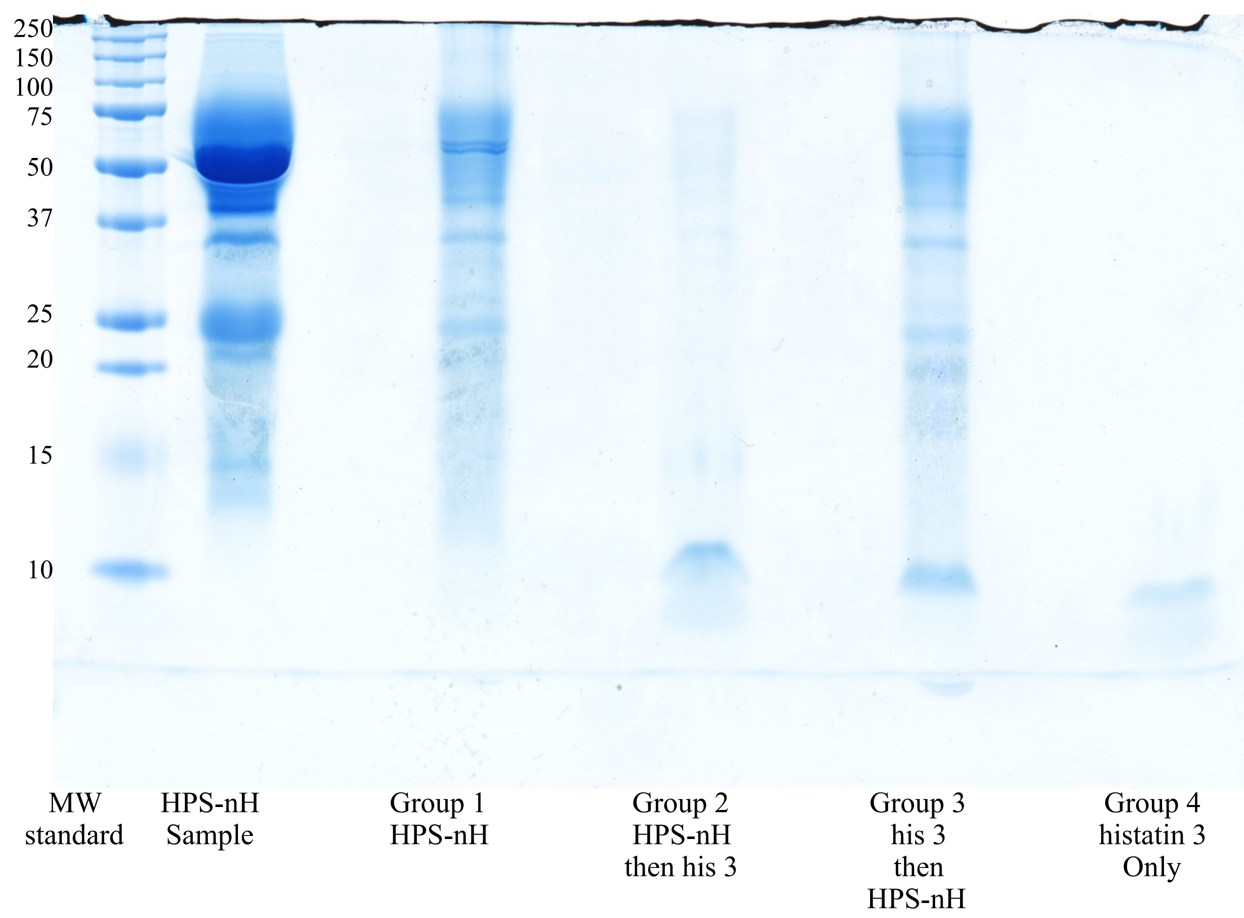

Figure 5 - Assessment of the third sample of groups 1, 2, 3 and 4 compared to original parotid saliva.

2.3 DISCUSSION

This is the first study evaluating bracket pellicle modulation via exposure to histatin 3. This is an area of particular interest in orthodontics due to the incidence of new white spot lesions occurring during orthodontic treatment. It is reported that 30-70% of patients undergoing orthodontic treatment develop white spot lesions.[1, 2] Histatin is a peptide that demonstrates antimicrobial properties, buffering capacity, as well as protection from demineralization.[13]

The assessment of the SDS-PAGE comparing each group demonstrates the differences between each group as well as the similarities between each triplicate sample. Staining in lanes representing groups 1-4 demonstrate proteins identified according to molecular weight (Figures 3-5). Proteins with higher molecular weight are represented at the top of each lane and progress proteins with low molecular weight at the bottom of each lane. The darker staining within each lane is representative of the number of proteins identified at the given molecular weight. While the individual proteins are not identified within the gel, the differences between each group justified further exploration with mass spectrometry.

Samples in each group were analyzed using mass spectrometry to identify both quantity and quality of proteins found within the pellicle formed on orthodontic brackets in each group. The number of proteins varied between each of the triplicates within each group, However, when considering only the proteins present in at least two of the three sample groups, the variation in the number of proteins decreased.

Histatin 3 was identified in two of the three samples in group 4, confirming the ability of histatin 3 to adhere to metallic orthodontic brackets.

Thirty nine individual proteins were identified by mass spectrometry in the group exposed to parotid saliva without histatins. Eighteen individual proteins were identified in group 2 where brackets were subjected first to parotid saliva without histatins, then to histatin 3. Twenty-one proteins were identified in group 3 which brackets were exposed to histatin 3 first, followed by parotid saliva without histatins. It is evident that exposure to histatin 3, either before or after exposure to parotid saliva without histatins modulates the quantity of proteins found in the acquired bracket pellicle.

When identified proteins are grouped based on their possible role in the acquired bracket pellicle structure formation, proteins can be divided into three main groups. The first group, consisting of proteins that have the ability to bind to metals (including phosphates and calcium). Binding to metals may provide insight to adhesion to the orthodontic bracket, while binding to phosphates and calcium may provide functionality in buffering and remineralization. The second group represents proteins that have been described to interact with other proteins, potentially involved in the formation of successive protein layers within the pellicle[13].

Proteins identified in the acquired bracket pellicle can also be grouped according to generally considered biological functions such as antimicrobial activities, immune response, remineralization, inflammatory process, buffering activity, and lubrication.[13, 52, 54]

Comparing the protein composition of the acquired bracket pellicle in each group with respect to relevant structural properties showed minor differences between group. The percentage of metal binding proteins was 21% in group 1, 22% in group 2 and 22% in group 3. The percentage of proteins that play a role in protein/protein interaction was 40% in group 1, 33 % in group 2, and 39% in group 3 (Figure 6).

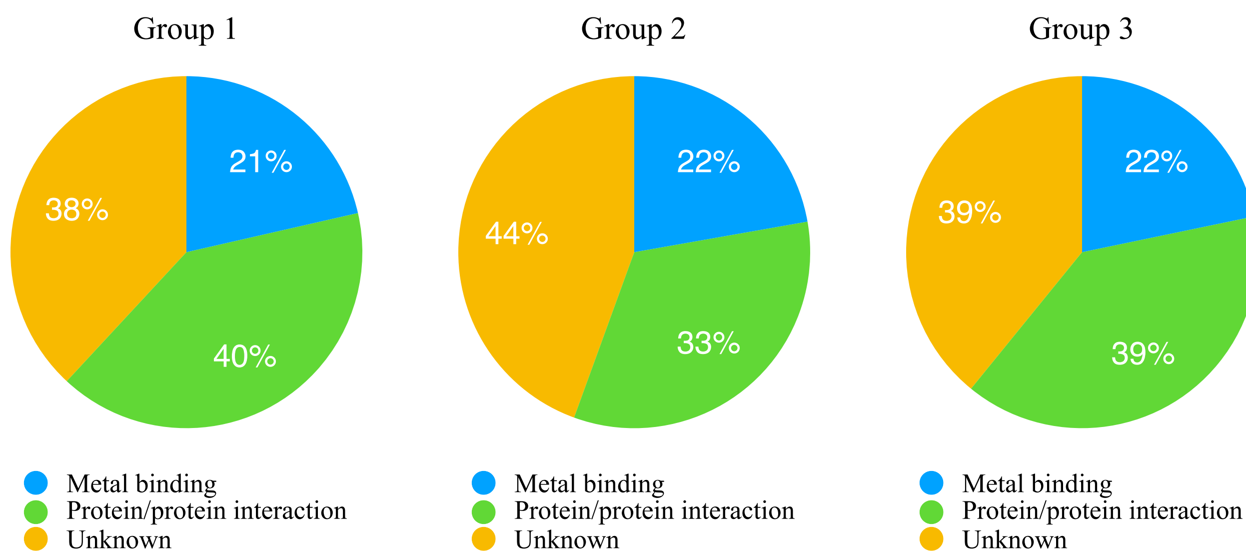

Figure 6 - Proteins identified in each group according to relevant structural properties.

Although the number of proteins varied in each group, the percentage of proteins according to relevant structural properties for each group was similar. This is likely due to the multi-functional properties of proteins identified in the acquired bracket pellicle.

There were some significant differences however when comparing the protein composition of the acquired bracket pellicle with respect to biological function in each group. The percentage of proteins exhibiting antimicrobial properties essentially doubled from the control (group 1) to groups 2 and 3.

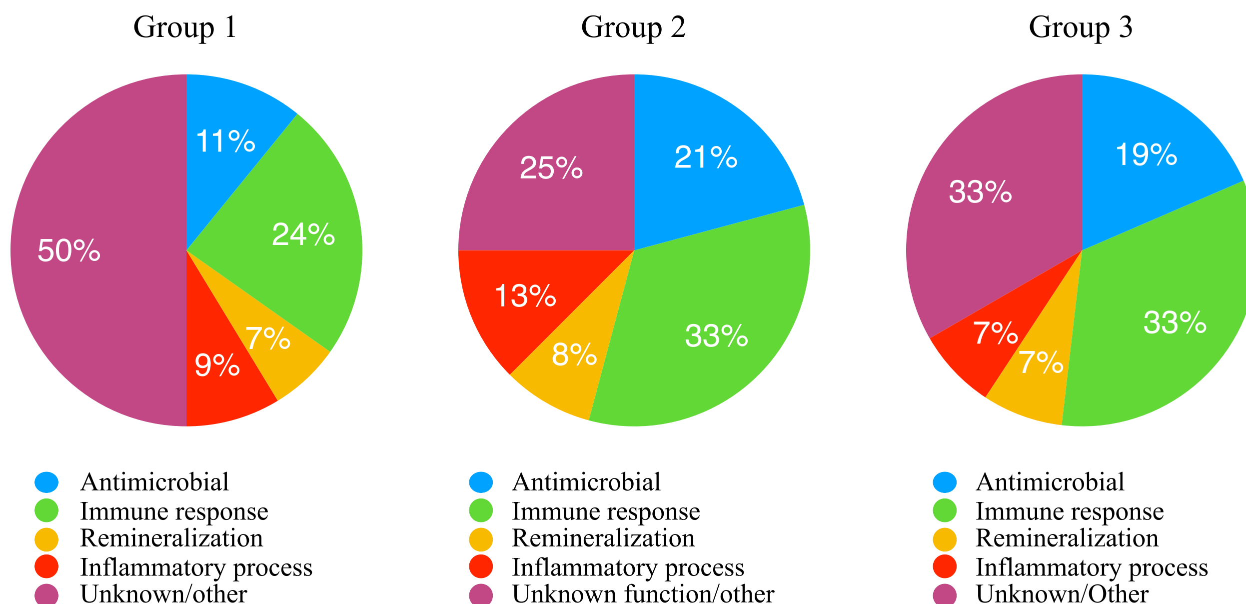

Figure 7 - Proteins identified in each group according to biologic function.

The percentage of proteins exhibiting functions relating to immune response also increased from 24% in the control, to 33% in groups 2 and 3. The percentage of proteins exhibiting remineralization properties however was relatively unchanged between all three groups (Figure 7).

The differences between the control (group 1) and groups 2 and , both in the number of proteins and their biologic function demonstrate the potential beneficial modulation of histatin 3 on the acquired bracket pellicle. Groups 2 and 3 showed differences in both the number of proteins and their biologic functions relative to the control, however when comparing groups 2 and 3 to each other, there were only minor differences. This is significant as pellicle modulation from exposure to histatin 3 could be introduced clinically in the form of an intra-oral rinse after pellicle formation.

2.4 CONCLUSIONS

- Significant differences in the number of proteins identified were noted between the control (group 1) and groups 2 and 3, suggesting that exposure to histatin 3 may alter the pellicle formation on orthodontic brackets.
- Significant functional differences were noted between the control (group 1) and groups 2 and 3 including, an increase in the percentage of proteins exhibiting antimicrobial activities and functions relating to immune response.
- Differences were limited regrading relevant structural properties due to redundant characteristics of salivary proteins.
- Our research confirms that histatin 3 binds to orthodontic brackets

2.5 FUTURE WORK

Incidence of white spot lesions in orthodontics continues to be problematic for both clinicians and patients and further investigation in ways to mitigate or reduce early enamel carries is warranted. Future work could include further modulation of the pellicle formation on orthodontic brackets with exposure to various functional peptide domains. Additionally, investigation of potential variation in subsequent biofilm characteristics could be pursued.

Selective modulation of the bracket protein pellicle, and subsequent biofilm, could be pursued via targeted oral products or additional surface coating of brackets aimed to create a less cariogenic biofilm on the surface of orthodontic brackets.

2.6 REFERENCES

1. Enaia M, Bock N, Ruf S. White-spot lesions during multi-bracket appliance treatment: a challenge for clinical excellence. American Journal of Orthodontics and Dentofacial Orthopedics. 2011;140:17-24.

2. Heymann GC, Grauer D. A contemporary review of white spot lesions in orthodontics. Journal of Esthetic and Restorative Dentistry. 2013;25:85-95.

3. Featherstone JDB. The science and practice of caries prevention. The Journal of the American Dental Association. 2000;131:887-899.

4. Siqueira WL, Custodio W, McDonald EE. New insights into the composition and functions of the acquired enamel pellicle. Journal of Dental Research. 2012;91:1110-1118.

5. Siqueira WL, Bakkal M, Xiao Y, Sutton JN, Mendes FM. Quantitative proteomic analysis of the effect of fluoride on the acquired enamel pellicle. PloS One. 2012;7:e42204.

6. Gastel J, Quirynen M, Teughels W, Pauwels M, Coucke W, Carels C. Microbial adhesion on different bracket types in vitro. The Angle Orthodontist. 2009;79:915-921.

7. Ryu H-S, Bae I-H, Lee K-G, Hwang H-S, Lee K-H, Koh J-T, Cho J-H. Antibacterial effect of silver-platinum coating for orthodontic appliances. The Angle Orthodontist. 2011;82:151-157.

8. Ahn S-J, Kho H-S, Lee S-W, Nahm D-S. Roles of salivary proteins in the adherence of oral streptococci to various orthodontic brackets. Journal of Dental Research. 2002;81:411-415.

9. do Nascimento LEAG, Pithon MM, dos Santos RL et al. Colonization of Streptococcus mutans on esthetic brackets: self-ligating vs conventional. American Journal of Orthodontics and Dentofacial Orthopedics. 2013;143:72-77.

10. Lee S-J, Kho H-S, Lee S-W, Yang W-S. Experimental salivary pellicles on the surface of orthodontic materials. American Journal of Orthodontics and Dentofacial Orthopedics. 2001;119:59-66.

11. Canales MP, Siqueira WL, Crosara K, Martins C. Binding of Salivary Proteins to Orthodontic Brackets [Unpublished Masters Thesis]. London, ON: Western University; 2018.

12. Siqueira WL, Oppenheim FG. Small molecular weight proteins/peptides present in the in vivo formed human acquired enamel pellicle. Archives of Oral Biology. 2009;54:437-444.

13. Siqueira WL, Zhang W, Helmerhorst EJ, Gygi SP, Oppenheim FG. Identification of protein components in in vivo human acquired enamel pellicle using LC− ESI− MS/MS. Journal of Proteome Research. 2007;6:2152-2160.

14. Siqueira WL, Margolis HC, Helmerhorst EJ, Mendes FM, Oppenheim FG. Evidence of intact histatins in the in vivo acquired enamel pellicle. Journal of Dental Research. 2010;89:626-630.

15. Oppenheim FG, Xu T, McMillian FM et al. Histatins, a novel family of histidine-rich proteins in human parotid secretion. Isolation, characterization, primary structure, and fungistatic effects on Candida albicans. Journal of Biological Chemistry. 1988;263:7472-7477.

16. Xu T, Levitz SM, Diamond RD, Oppenheim FG. Anticandidal activity of major human salivary histatins. Infection and Immunity. 1991;59:2549-2554.

17. Pollock JJ, Denepitiya L, MacKay BJ, Iacono VJ. Fungistatic and fungicidal activity of human parotid salivary histidine-rich polypeptides on Candida albicans. Infection and Immunity. 1984;44:702-707.

18. Featherstone JD. Dental caries: a dynamic disease process. Aust Dent J. 2008;53:286-291.

19. Hamdan AM, Maxfield BJ, Tüfekçi E, Shroff B, Lindauer SJ. Preventing and treating white-spot lesions associated with orthodontic treatment: a survey of general dentists and orthodontists. The Journal of the American Dental Association. 2012;143:777-783.

20. Rosenbloom RG, Tinanoff N. Salivary Streptococcus mutans levels in patients before, during, and after orthodontic treatment. American Journal of Orthodontics and Dentofacial Orthopedics. 1991;100:35-37.

21. Eliades T, Eliades G, Brantley WA. Microbial attachment on orthodontic appliances: I. Wettability and early pellicle formation on bracket materials. American Journal of Orthodontics and Dentofacial Orthopedics. 1995;108:351-360.

22. Humphrey SP, Williamson RT. A review of saliva: normal composition, flow, and function. The Journal of Prosthetic Dentistry. 2001;85:162-169.

23. Edgar WM, O'Mullane DM, Dawes C. Saliva and oral health. British Dental Association London; 2012

24. Siqueira WL, Dawes C. The salivary proteome: challenges and perspectives. PROTEOMICS–Clinical Applications. 2011;5:575-579.

25. Siqueira WL, Salih E, Wan DL, Helmerhorst EJ, Oppenheim FG. Proteome of human minor salivary gland secretion. Journal of Dental Research. 2008;87:445-450.

26. Dawes C, Wood CM. The contribution of oral minor mucous gland secretions to the volume of whole saliva in man. Archives of Oral Biology. 1973;18:337-342.

27. Rudney JD. Does variability in salivary protein concentrations influence oral microbial ecology and oral health. Critical Reviews in Oral Biology & Medicine. 1995;6:343-367.

28. Thaysen JH, Thorn NA, Schwartz IL. Excretion of sodium, potassium, chloride and carbon dioxide in human parotid saliva. American Journal of Physiology-Legacy Content. 1954;178:155-159.

29. Turner RJ, Sugiya H. Understanding salivary fluid and protein secretion. Oral diseases. 2002;8:3-11.

30. Roth GI, Calmes RB. Oral Biology. Mosby; 1981

31. Veerman ECI, Van den Keybus PAM, Vissink A, Amerongen AVN. Human glandular salivas: their separate collection and analysis. European Journal of Oral Sciences. 1996;104:346-352.

32. Cabras T, Pisano E, Boi R et al. Age-dependent modifications of the human salivary secretory protein complex. Journal of Proteome Research. 2009;8:4126-4134.

33. Moss SJ. Clinical implications of recent advances in salivary research. Journal of Esthetic and Restorative Dentistry. 1995;7:197-203.

34. Mandel ID. The functions of saliva. Journal of Dental Research. 1987;66:623-627.

35. Tabak LA, Levine MJ, Mandel ID, Ellison SA. Role of salivary mucins in the protection of the oral cavity. Journal of Oral Pathology & Medicine. 1982;11:1-17.

36. Mandel ID. Impact of saliva on dental caries. Compendium (Newtown, Pa) Supplement. 1989S476-81.

37. Levine MJ. Development of artificial salivas. Critical Reviews in Oral Biology & Medicine. 1993;4:279-286.

38. Messana I, Inzitari R, Fanali C, Cabras T, Castagnola M. Facts and artifacts in proteomics of body fluids. What proteomics of saliva is telling us. Journal of Separation Science. 2008;31:1948-1963.

39. Bennick A. Salivary proline-rich proteins. Molecular and Cellular Biochemistry. 1982;45:83-99.

40. Oppenheim FG, Salih E, Siqueira WL, Zhang W, Helmerhorst EJ. Salivary proteome and its genetic polymorphisms. Ann N Y Acad Sci. 2007;1098:22-50.

41. Melino S, Santone C, Di Nardo P, Sarkar B. Histatins: salivary peptides with copper(II)- and zinc(II)-binding motifs: perspectives for biomedical applications. FEBS J. 2014;281:657-672.

42. Basiri T, Johnson ND, Moffa EB et al. Duplicated or hybridized peptide functional domains promote oral homeostasis. Journal of Dental Research. 2017;96:1162-1167.

43. Trindade F, Amado F, da Costa JP et al. Salivary peptidomic as a tool to disclose new potential antimicrobial peptides. Journal of Proteomics. 2015;115:49-57.

44. Bobek LA, Aguirre A, Levine MJ. Human salivary cystatin S. Cloning, sequence analysis, hybridization in situ and immunocytochemistry. Biochemical Journal. 1991;278:627-635.

45. Lamkin MS, Oppenheim FG. Structural features of salivary function. Critical Reviews in Oral Biology & Medicine. 1993;4:251-259.

46. Arhakis A, Karagiannis V, Kalfas S. Salivary alpha-amylase activity and salivary flow rate in young adults. The Open Dentistry Journal. 2013;7:7.

47. Zakowski JJ, Bruns DE. Biochemistry of human alpha amylase isoenzymes. CRC Critical Reviews in Clinical Laboratory Sciences. 1985;21:283-322.

48. Scannapieco FA, Bergey EJ, Reddy MS, Levine MJ. Characterization of salivary alpha-amylase binding to Streptococcus sanguis. Infection and Immunity. 1989;57:2853-2863.

49. Tabak LA. In defense of the oral cavity: structure, biosynthesis, and function of salivary mucins. Annual Review of Physiology. 1995;57:547-564.

50. Bruno LS, Li X, Wang L et al. Two-hybrid analysis of human salivary mucin MUC7 interactions. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research. 2005;1746:65-72.

51. Yao Y, Grogan J, Zehnder M et al. Compositional analysis of human acquired enamel pellicle by mass spectrometry. Archives of Oral Biology. 2001;46:293-303.

52. Hannig M, Joiner A. The structure, function and properties of the acquired pellicle. The teeth and their environment. Monogr Oral Sci. 2006;19:29-64.

53. Li J, Helmerhorst EJ, Leone CW et al. Identification of early microbial colonizers in human dental biofilm. Journal of Applied Microbiology. 2004;97:1311-1318.

54. Lendenmann U, Grogan J, Oppenheim FG. Saliva and dental pellicle-a review. Advances in Dental Research. 2000;14:22-28.

55. Bradway SD, Bergey EJ, Jones PC, Levine MJ. Oral mucosal pellicle. Adsorption and transpeptidation of salivary components to buccal epithelial cells. Biochemical Journal. 1989;261:887-896.

56. Edgerton M, Lo SE, Scannapieco FA. Experimental salivary pellicles formed on titanium surfaces mediate adhesion of streptococci. International Journal of Oral and Maxillofacial Implants. 1996;11:443-449.

57. Gibbons RJ. Bacterial adhesion to oral tissues: a model for infectious diseases. Journal of Dental Research. 1989;68:750-760.

58. Mozes N, Marchal F, Hermesse MP et al. Immobilization of microorganisms by adhesion: interplay of electrostatic and nonelectrostatic interactions. Biotechnology and Bioengineering. 1987;30:439-450.

59. Fournier A, Payant L, Bouclin R. Adherence of Streptococcus mutans to orthodontic brackets. American Journal of Orthodontics and Dentofacial Orthopedics. 1998;114:414-417.

60. Lim B-S, Lee S-J, Lee J-W, Ahn S-J. Quantitative analysis of adhesion of cariogenic streptococci to orthodontic raw materials. American Journal of Orthodontics and Dentofacial Orthopedics. 2008;133:882-888.

61. Carlen A, Börjesson A-C, Nikdel K, Olsson J. Composition of pellicles formed in vivo on tooth surfaces in different parts of the dentition, and in vitro on hydroxyapatite. Caries Research. 1998;32:447-455.

62. O'Farrell PH. High resolution two-dimensional electrophoresis of proteins. Journal of Biological Chemistry. 1975;250:4007-4021.

63. Klose J. Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues. Humangenetik. 1975;26:231-243.

64. Yao Y, Berg EA, Costello CE, Troxler RF, Oppenheim FG. Identification of protein components in human acquired enamel pellicle and whole saliva using novel proteomics approaches. Journal of Biological Chemistry. 2003;278:5300-5308.

65. Peng J, Gygi SP. Proteomics: the move to mixtures. Journal of Mass Spectrometry. 2001;36:1083-1091.

66. Han X, Aslanian A, Yates III JR. Mass spectrometry for proteomics. Current Opinion in Chemical Biology. 2008;12:483-490.

67. Flora B, Gusman H, Helmerhorst EJ, Troxler RF, Oppenheim FG. A new method for the isolation of histatins 1, 3, and 5 from parotid secretion using zinc precipitation. Protein Expression and Purification. 2001;23:198-206.

68. Wijkstrom-Frei C, El-Chemaly S, Ali-Rachedi R et al. Lactoperoxidase and human airway host defense. American Journal of Respiratory Cell and Molecular Biology. 2003;29:206-212.

TABLES

Table 2 - Proteins identified in at least two of three samples in group 1

Accession Number	Protein name	Biologic function	Chemical Properties
P25311	Zinc-alpha-2-glycoprotein	Immune response	Unknown
Q96Q06	Perilipin-4	Unknown	Unknown
P0DOY2	Immunoglobulin lambda constant 2	Immune response, inflammation	Protein/protein interaction
P02814	Submaxillary gland androgen-regulated protein 3B	Unknown	Protein/protein interaction
B7Z759	cDNA FLJ61672, highly similar to Proteoglycan-4 (Fragment)	Immune response	Protein/protein interaction
Q7Z5P9	Mucin-19	Lubrication	Unknown
Q12955	Ankyrin-3	Unknown	Protein/protein interaction
E9PKG6	Nucleobindin-2	Mineralization	Metal Binding, Calcium
A0A0C4DGN4	Zymogen granule protein 16 homolog B	Unknown	Unknown
P01876	Immunoglobulin heavy constant alpha 1	Antimicrobial, Immune response, inflammation	Unknown
F5H386	Lactoperoxidase	Antimicrobial, Immune response	Protein/protein interaction
Q9UGM3	Deleted in malignant brain tumors 1 protein	Antimicrobial, Immune response	Protein/protein interaction, metal binding, Calcium
Q96DR5	BPI fold-containing family A member 2	Antimicrobial, Immune response	Protein/protein interaction
P01833	Polymeric immunoglobulin receptor	Inflammation	Unknown
P12273	Prolactin-inducible protein	Immune response, antimicrobial	Unknown

Accession Number	Protein name	Biologic function	Chemical Properties
P23280	Keratin 1	Unknown	Unknown
P23280	Carbonic anhydrase 6	Unknown	Metal Binding
Q8WXI7	Mucin-16	Lubrication	Unknown
P04745	Alpha-amylase 1	Mineralization	Metal Binding
B2RBH0	cDNA, FLJ95505, highly similar to Homo sapiens leucine rich repeat containing 2 (LRRC2), mRNA	Unknown	Unknown
P35527	Keratin, type I cytoskeletal 9	Unknown	Unknown
	cAMP-dependent protein kinase catalytic subunit beta (Fragment)	Unknown	Protein/protein interaction
P49790	Nuclear pore complex protein Nup153	Immune response	Protein/protein interaction
Q59FZ5	PTPRF interacting protein alpha 1 isoform b variant (Fragment)	Unknown	Unknown
E7ETD6	Nucleosome-remodeling factor subunit BPTF (Fragment)	Unknown	Metal Binding
Q8IVL0	Neuron navigator 3	Immune response, inflammation	Protein/protein interaction
P35908	Keratin, type II cytoskeletal 2 epidermal	Unknown	Protein/protein interaction
P01834	Immunoglobulin kappa constant	Immune response	Unknown
A0A1B0GV45	Unconventional myosin-XVIIIa (Fragment)	Unknown	Unknown
E9PAV3	Nascent polypeptide-associated complex subunit alpha, muscle-specific form	Unknown	Protein/protein interaction
Q8WZ42	Titin	Unknown	Protein/protein interaction, metal binding
Q8IZF6	Adhesion G-protein coupled receptor G4	Unknown	Protein/protein interaction

Accession Number	Protein name	Biologic function	Chemical Properties
Q9C0D5	Protein TANC1	Unknown	Unknown
H7C1L9	E3 ubiquitin-protein ligase TRIP12 (Fragment)	Unknown	Unknown
E7EWQ5	Microtubule-associated serine/threonine-protein kinase 4	Unknown	Protein/protein interaction, metal binding
A0A087X011	Kinesin-like protein	Unknown	Protein/protein interaction
Q9H040	SprT-like domain-containing protein Spartan	Unknown	Protein/protein interaction, metal binding, Zinc
Q9Y6V0	Protein piccolo	Mineralization	Metal Binding

Table 3 - Proteins identified in at least two of three samples in group 2

Accession Number	Protein name	Biologic function	Chemical Properties
P01834	Immunoglobulin kappa constant	Immune response	Unknown
Q9UGM3	Deleted in malignant brain tumors 1 protein	Antimicrobial, Immune response	Protein/protein interaction, metal binding, Calcium
P0DOY2	Immunoglobulin lambda constant 2	Immune response, inflammation	Protein/protein interaction
A0A0C4DGN4	Zymogen granule protein 16 homolog B	Unknown	Unknown
P23280	Carbonic anhydrase 6	Unknown	Metal binding
Q96DR5	BPI fold-containing family A member 2	Antimicrobial, Immune response	Protein/protein interaction
P01833	Polymeric immunoglobulin receptor	Inflammation	Unknown

Accession Number	Protein name	Biologic function	Chemical Properties
P12273	Prolactin-inducible protein	Immune response, antimicrobial	Unknown
P04745	Alpha-amylase 1	Mineralization	Metal binding
P35908	Keratin, type II cytoskeletal 2 epidermal	Unknown	Protein/protein interaction
P35527	Keratin, type I cytoskeletal 9	Unknown	Unknown
H6VRF8	Keratin 1	Unknown	Unknown
E9PLR0	Nucleobindin-2	Mineralization	Metal binding, Calcium
P02814	Submaxillary gland androgen-regulated protein 3B	Unknown	Protein/protein interaction
P25311	Zinc-alpha-2-glycoprotein	Immune response	Protein/protein interaction
B4E1M1	cDNA FLJ60391, highly similar to Lactoperoxidase (EC 1.11.1.7)	Antimicrobial, Immune response	Unknown
P01876	Immunoglobulin heavy constant alpha 1	Antimicrobial, Immune response, inflammation	Unknown

Table 4 - Proteins identified in at least two of three samples in group 3

Accession Number	Protein name	Biologic function	Chemical Properties
P01834	Immunoglobulin kappa constant	Immune response	Unknown
P25311	Zinc-alpha-2-glycoprotein	Immune response	Protein/protein interaction
Q9UGM3	Deleted in malignant brain tumors 1 protein	Antimicrobial, Immune response	Protein/protein interaction, metal binding, Calcium
A0A0C4DGN4	Zymogen granule protein 16 homolog B	Unknown	Unknown

Accession Number	Protein name	Biologic function	Chemical Properties
P01876	Immunoglobulin heavy constant alpha 1	Antimicrobial, Immune response, inflammation	Unknown
P02814	Submaxillary gland androgen-regulated protein 3B	Unknown	Protein/protein interaction
F5H386	Lactoperoxidase	Antimicrobial, Immune response	Protein/protein interaction
Q8WXI7	Mucin-16	Lubrication	Unknown
Q96DR5	BPI fold-containing family A member 2	Antimicrobial, Immune response	Protein/protein interaction
P01833	Polymeric immunoglobulin receptor	Inflammation	Unknown
P12273	Prolactin-inducible protein	Immune response, antimicrobial	Unknown
P04745	Alpha-amylase 1	Mineralization	Metal binding
P35527	Keratin, type I cytoskeletal 9	Unknown	Unknown
H6VRF8	Keratin 1	Unknown	Unknown
Q9Y6V0	Protein piccolo	Mineralization	Metal Binding
P13645	Keratin, type I cytoskeletal 10	Unknown	Protein/protein interaction
A0M8Q6	Immunoglobulin lambda constant 7	Immune response	Protein/protein interaction
O75592	E3 ubiquitin-protein ligase MYCBP2	Unknown	Protein/protein interaction, metal binding, Zinc
Q7Z5P9	Mucin-19	Lubrication	Unknown
B7Z4R8	cDNA FLJ53364, highly similar to Proteoglycan-4 (Fragment)	Immune response	Protein/protein interaction
P23280	Carbonic anhydrase 6	Unknown	Metal binding

Table 5 - Proteins identified in at least two of three samples in group 4

Accession Number	Protein name
P13645	Keratin, type I cytoskeletal 10
B4DRR0	cDNA FLJ53910, highly similar to Keratin, type II cytoskeletal 6A
P02533	Keratin, type I cytoskeletal 14
P35908	Keratin, type II cytoskeletal 2 epidermal
P35527	Keratin, type I cytoskeletal 9
B4E1T1	cDNA FLJ54081, highly similar to Keratin, type II cytoskeletal 5
H6VRF8	Keratin 1
B7ZMD7	Alpha-amylase
P08779	Keratin, type I cytoskeletal 16
X6RAH8	Histatin-3
A6NN14	Zinc finger protein 729

Table 6 - Proteins identified in groups 1, 2 and 3

Accession Number	Protein name	Biologic function	Chemical Properties
P01834	Immunoglobulin kappa constant	Immune response	Unknown
P25311	Zinc-alpha-2-glycoprotein	Immune response	Protein/protein interaction
Q9UGM3	Deleted in malignant brain tumors 1 protein	Antimicrobial, Immune response	- protein/protein interaction - Ca2+ binding
P23280	Carbonic anhydrase 6	Unknown	Metal binding
Q96DR5	BPI fold-containing family A member 2	Antimicrobial, Immune response	Protein/protein interaction
P01833	Polymeric immunoglobulin receptor	Inflammatory process	Unknown
P12273	Prolactin-inducible protein	Immune response, antimicrobial - Binds to streptococci in mice	Unknown
A0A0C4 DGN4	Zymogen granule protein 16 homolog B	Unknown	Unknown
P04745	Alpha-amylase 1	Remineralization, unknown	Metal binding
P01876	Immunoglobulin heavy constant alpha 1	Antimicrobial, Immune response, inflammation	Unknown
P35527	Keratin, type I cytoskeletal 9	Karatinization	Unknown
P02814	Submaxillary gland androgen-regulated protein 3B	Unknown	Protein/protein interaction
H6VRF8	Keratin 1	Unknown	Unknown

Table 7 - Proteins identified in groups 1 and 2

Accession Number	Protein name	Biologic function	Chemical Properties
P0DOY2	Immunoglobulin lambda constant 2	Immune response, inflammation	protein/protein interaction
E9PKG6	Nucleobindin-2	Remineralization	Metal Calcium ion binding
P35908	Keratin, type II cytoskeletal 2 epidermal	Unknown	protein/protein interaction

Table 8 - Proteins identified in groups 1 and 3

Accession Number	Protein name	Biologic function	Chemical Properties
F5H386	Lactoperoxidase	Antimicrobial, Immune response	protein/protein interaction
Q7Z5P9	Mucin-19	Lubrication	Unknown
Q8WXI7	Mucin-16	Lubrication	Unknown
Q9Y6V0	Protein piccolo	ReMineralization	Metal binding

Table 9 - Proteins identified in groups 2 and 3

Accession Number	Protein name	Biologic function	Chemical Properties
P13645	Keratin, type I cytoskeletal 10	Unknown	protein/protein interaction

Table 10 - Proteins unique to group 1

Accession Number	Protein name	Biologic function	Chemical Properties
B7Z759	cDNA FLJ61672,highly similar to Proteoglycan-4 (Fragment)	Immune response	Protein/protein interaction
Q12955	Ankyrin-3	Unknown	protein/protein interaction
A0A1B0GV45	Unconventional myosin-XVIIIa (Fragment)	Unknown	Unknown
E9PAV3	Nascent polypeptide-associated complex subunit alpha, muscle-specific form	Unknown	protein/protein interaction
Q9C0D5	Protein TANC1	Unknown	Unknown
Q59FZ5	PTPRF interacting protein alpha 1 isoform b variant (Fragment)	Unknown	Unknown

Accession Number	Protein name	Biologic function	Chemical Properties
P15822	Zinc finger protein 40	Unknown	protein/protein interaction Metal ion binding
Q8WZ42	Titin	Unknown	protein/protein interaction Metal ion binding
B1APF8	cAMP-dependent protein kinase catalytic subunit beta (Fragment)	Unknown	protein/protein interaction
Q8IVL0	Neuron navigator 3	Inflammation Immune response	protein/protein interaction
O15021	Microtubule-associated serine/threonine-protein kinase 4	Unknown	Metal binding, protein/protein interaction
B2RBH0	cDNA, FLJ95505, highly similar to Homo sapiens leucine rich repeat containing 2 (LRRC2), mRNA	Unknown	Unknown
A0A087X011	Kinesin-like protein	Unknown	protein/protein interaction
H7C2Y1	E3 ubiquitin-protein ligase TRIP12 (Fragment)	Unknown	Unknown
Q8IZF6	Adhesion G-protein coupled receptor G4	Unknown	protein/protein interaction
Q96Q06	Perilipin-4	Unknown	Unknown
Q9H040	SprT-like domain-containing protein Spartan	Unknown	protein/protein interaction, zinc binding

Accession Number	Protein name	Biologic function	Chemical Properties
P49790	Nuclear pore complex protein Nup153	Immune response	protein/protein interaction
F5GXF5	Nucleosome-remodeling factor subunit BPTF (Fragment)	Unknown	Metal binding

Table 11 - Proteins unique to group 2

Accession Number	Protein name	Biologic function	Chemical Properties
B4E1M1	cDNA FLJ60391, highly similar to Lactoperoxidase (EC 1.11.1.7)	Antimicrobial, Immune response	Unknown

Table 12 - Proteins unique to group 3

Accession Number	Protein name	Biologic function	Chemical Properties
A0M8Q6	Immunoglobulin lambda constant 7	Immune response	Protein/protein interaction
O75592	E3 ubiquitin-protein ligase MYCBP2	Unknown	protein/protein interaction, zinc-binding
B7Z4R8	cDNA FLJ53364, highly similar to Proteoglycan-4 (Fragment)	Immune response	Protein/protein interaction

APPENDICES

Appendix 1 - Solutions

Gel Percentage (4%)	
Stacking	**Volume (mL)**
30% Polyacrylamide	0.850
1.5M Tris (pH 6.8)	0.625
10% SDS	0.050
Ultra Pure Mili Q Water	3.4
10% Ammonium	0.050
TEMED	0.005

Gel Percentage (12%)	
Steparating	**Volume (mL)**
30% Polyacrylamide	6.000
1.5M Tris (pH 8.8)	3.750
10% SDS	0.150
Ultra Pure Mili Q Water	5.000
10% Ammonium	0.150
TEMED	0.006

Running Buffer (pH 8.3)	
Tris base	30.2 g
1.5M Tris (pH 8.8)	188.0 g
10% SDS	100 mL

Sample Buffer	
1M Tris/HCL, pH 6.8	0.6 mL
Glycerol	2.5 mL
10% SDS	2 mL
1% Bromophenol Blue	1 mL
2 Mercaptoethanol	0.5 mL
Ultra Pure Mili Q Water	10 mL

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Dental Research Award

Saskatchewan Chapter of the Canadian Society of Dentistry for Children Book Award